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Pyruvate dehydrogenase activity is important for colonization of seeds and roots by *Enterobacter cloacae*

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Abstract

Enterobacter cloacae is a plant-beneficial bacterium that shows promise for suppression of damping-off of cucumber and other crops caused by *Pythium ultimum*. We have been using a mutational approach to determine the *E. cloacae* genes important in bacterial–plant and bacterial–pathogen interactions in the spermosphere and rhizosphere. *E. cloacae* M43 is a transposon mutant of *E. cloacae* 501R3 that was significantly impaired in colonization of seeds and roots of diverse crop plants. Strain M43 did not increase in population on cucumber, sunflower, and wheat seeds and was significantly reduced in growth on pea seeds relative to strain 501R3. Populations of M43 were also dramatically lower than those of strain 501R3 in cucumber, pea, sunflower, and wheat rhizosphere in 42 d experiments. Molecular characterization of M43 demonstrated that there was a single transposon insertion in the genome of this strain and that this insertion was in a region of the *E. cloacae* genome with a high degree of DNA sequence identity with *aceF*. *aceF* encodes the dihydrolipoamide acetyltransferase subunit of the pyruvate dehydrogenase complex (PDHC). Cell lysates from strain 501R3 grown on minimal medium plus 50 mM glycerol and 2 mM acetate contained 0.011 ± 0.0036 U pyruvate dehydrogenase activity while cell lysates from M43 grown under identical conditions contained no detectable pyruvate dehydrogenase activity. Additionally, the nutritional use profile of M43 under aerobic and anaerobic conditions was as expected for an *ace* mutant. Experiments reported here strongly suggest a role for *aceF* and the PDHC in colonization of seeds and roots of diverse crop plants by *E. cloacae*.

Keywords: Biological control; Colonization; Rhizosphere; Spermosphere

1. Introduction

Beneficial bacteria applied as treatments of seeds and other plant parts have been shown to suppress plant diseases caused by seed, root, and foliar pathogens (Larkin et al., 1998; Weller, 1988; Meyer and Roberts, 2002). Colonization of the spermosphere and rhizosphere is thought to be important for effective suppression of many of these pathogens (Weller, 1988; Bull et al., 1991; Chin-A-Woeng et al., 2000; Lugtenberg et al., 2001). A number of traits, including growth by beneficial bacteria, have been shown to be important for colonization (Mazzola et al., 1992; Rainey, 1999; Lugtenberg et al., 2001; de Weert et al., 2002; Lohrke et al., 2002; Martínez-Granero et al., 2005). Seeds and roots support growth and other activities by microbes through the release of a complex mixture of carbohydrates, amino acids, organic acids, and other nutrients (Kraffczyk et al., 1984; Lynch and Whipps, 1990). However, there is a significant gap in our understanding of the roles played by genes and catabolic pathways functioning in beneficial bacteria, and the nutrients released by plant seeds and roots during colonization (Roberts et al., 1999; Lohrke et al., 2002).

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Enterobacter cloacae is a beneficial, enteric bacterium capable of suppressing damping-off of cucumber and other crops caused by *Pythium ultimum* (Hadar et al., 1983; Nelson et al., 1986; Howell et al., 1988; Nelson, 1988). This bacterium is also an effective colonizer of the spermosphere and rhizosphere of a number of plant species (Kleeberger et al., 1983; Nelson, 1988; Roberts et al., 1999; Lohrke et al., 2002). Our approach has been to inactivate genes functioning in pathways that metabolize reduced carbon in *E. cloacae* 501R3 and subsequently determine the importance of these genes to colonization of seeds and roots by this bacterium (Roberts et al., 1996, 1999; Lohrke et al., 2002). This approach also provides insight into the role that plant nutrients metabolized by these pathways have in colonization. *E. cloacae* mutants, each containing a sole mini-Tn*5* Km transposon insertion in *pfkA*, *sdhA*, *rpiA*, or *cyaA*, were shown to be reduced in colonization of seeds and roots of certain plants demonstrating a role for each of these genes, and the pathways they function in, in colonization (Roberts et al., 1996, 1999, 2007; Lohrke et al., 2002; Liu et al., submitted). *pfkA* functions in the Embden–Meyerhof–Parnas pathway (glycolysis) while *sdhA* and *rpiA* function in the TCA cycle and the Pentose Phosphate Pathway, respectively (Roehl and Vinopal, 1976; Sprenger, 1995; Cecchini et al., 2002). *cyaA* encodes adenylate cyclase, the enzyme that catalyzes the synthesis of the regulatory molecule cyclic AMP. Cyclic AMP, when complexed with the Cyclic AMP Regulatory Protein, regulates expression of a number of genes involved in catabolism and other key cellular processes (Botsford and Harman, 1992; Zheng et al., 2004). We report here the isolation of the mutant *E. cloacae* M43 and demonstrate that this mutant contains a sole mini-Tn*5* Km transposon insertion in *aceF*, a gene that encodes a subunit of the pyruvate dehydrogenase complex (PDHC). In enteric bacteria such as *E. cloacae*, PDHC converts pyruvate, generated by the Embden–Meyerhof–Parnas pathway, to acetyl-CoA, which is metabolized by the TCA cycle, and is critical for metabolism of carbohydrates, amino acids, and TCA cycle intermediates under aerobic conditions (Cassey et al., 1998). We also show that *E. cloacae* M43 is dramatically reduced in colonization of the seeds and roots of diverse crop plants.

2. Materials and methods

2.1. Bacterial strains and plasmids

A description of bacterial strains and plasmids is in Table 1. Unless stated otherwise, strains were grown at 35 °C on Luria–Bertani (LB) broth (Miller, 1972) or agar or M56 salts (Carlton and Brown, 1981) broth or agar. Broth cultures were shaken at 250 rev min^{-1}. Culture media were supplemented with 50 μg ampicillin (Ap) ml^{-1}, 75 μg kanamycin (Kan) ml^{-1}, or 100 μg rifampicin (Rif) ml^{-1} where appropriate.

Table 1
Bacterial strains, plasmids, and DNA primers

Strain, plasmid, or primer	Relevant characteristics[a]	Reference or source
Strains		
Enterobacter cloacae 501R3	Spontaneous Rif^R mutant of *E. cloacae* EcCT501	Roberts et al. (1992)
E. cloacae M43	Mini-Tn*5* Km mutant of 501R3; *aceF*::mini-Tn*5* Km; Rif^R, Kan^R	This study
Escherichia coli DH5α	(Φ80d*lacZ*ΔM15) Δ(*lacZYA-argF*) *U169 glnV44 deoR gyrA96 recA1 relA91 endA1 thi-1 hsdR17*	Sambrook and Russell (2001)
Plasmids		
pGEM-7Zf(+)	Ap^R; cloning vector	Promega
pM43	Ap^R, Kan^R; contains an approximately 3-kb *Kpn*I fragment from strain M43 containing *aceF*::mini-Tn*5* Km	This study
Primers		
MT5R 2906	GGG CCT TGA TGT TAC CGA GAG C	Lohrke et al. (2002)
MT5L 294	TAA GCG TGC ATA ATA AGC CCT ACA	Lohrke et al. (2002)
E1_1136F	CGC AAT CAC CAA ATT CAA	This study
E2R2:	AGT CCC TTT CAC TTT CGC CAG	This study
E2 F3:	CCA AAG CGG AAG GCA AAT CTG	This study
LPDR	GCA ACG GAA AGC GGC AGA ATA	This study

[a] Ap^R, ampicillin resistant; Kan^R, kanamycin resistant; Rif^R, rifampicin resistant.

2.2. Molecular techniques

DNA isolations, transformations, electroporation, restriction digests, electrophoresis, ligations, and Southern blot hybridizatons were performed as described previously (Sambrook and Russell, 2001). Plasmid pM43 was constructed by digesting *E. cloacae* M43 genomic DNA with *Bgl*II followed by ligation to *Bgl*II-digested pGEM-7Zf(+). Plasmid pM43 was isolated after electroporation of this ligation mixture into *Escherichia coli* DH5α and selection on LB agar containing Ap and Kan.

The nucleotide sequence of both strands of the *E. cloacae* portion of pM43 was obtained using PCR-mediated Taq DyeDeoxy terminator cycle sequencing. Primers designed to the right (MT5R 2096) and left (MT5L 294) ends of mini-Tn*5* Km were used (Table 1; Fig. 1) (Lohrke et al., 2002). Primers designed from prior sequencing runs were used in sequencing reactions directed at completing the sequence of the *E. cloacae* portion of pM43. Sequences were analyzed using the Lasergene DNA analysis program (DNAStar Inc., Madison, WI). PCR amplification and sequencing were used to compare DNA sequence in pM43

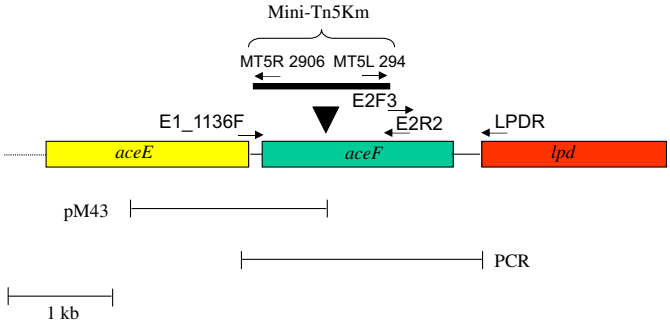

Fig. 1. Physical map of the portion of the *Enterobacter cloacae* M43 genome containing mini-Tn*5* Km. The location of *aceE*, *aceF*, and *lpd* are indicated by boxes and the direction of transcription of these genes is from left to right. The downward pointing arrow indicates the site of the mini-Tn*5* Km insertion. Primers for sequencing reactions are indicated by the horizontal arrows. Sequence was obtained from the *E. cloacae* portion of plasmid pM43 (pM43) or from PCR product (PCR). Features are approximately to scale. The nucleotide sequence and sequences of translated proteins have been deposited in GenBank under accession number EF151242.

with that in strains M43 and 501R3 and to obtain sequence of the downstream portion of *aceF* through the 5′ portion of *lpd* in 501R3 (Fig. 1). Primers used are listed in Table 1 and the strategy used is indicated in Fig. 1. PCRs were initiated with a 95 °C denaturation of 5 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s, and ending with a 15 min incubation at 72 °C. The PCR products were separated from reaction components on 1.5% NuSieve agarose gels, excised from the gels, frozen, and the DNA extruded from the gels by centrifugation. Nucleotide sequencing was performed with ABI BigDye 3.1 sequencing reagents (Applied Biosystems, Foster City, CA) and run on an Applied Biosystems 3100 Automated DNA Analyzer. Sequence data was edited and assembled with Sequencher (Gene Codes Corp., Ann Arbor, MI). BLAST searches of databases (Altschul et al., 1997) were conducted with translated sequences by using the Blastp program (National Center for Biotechnology Information web page, www.ncbi.nlm.nih.gov/BLAST). GeneContext searches were conducted with the Gene Context Tool (Ciria et al., 2004) on 12/10/2006 with 345 microbial genomes included.

2.3. Nucleotide sequence accession number

The nucleotide sequence and the sequences of the translated proteins have been deposited in GenBank under accession number EF151242.

2.4. Enzyme assays

For determination of pyruvate dehydrogenase activity, *E. cloacae* strains were incubated overnight in 250 ml Erlenmeyer flasks containing 50 ml M56 salts plus 50 mM glycerol and 2 mM acetate or 50 mM glucose and 2 mM acetate at 35 °C and 300 rev min^{-1}. Overnight cultures were diluted 1:5 with the same medium used for overnight growth and incubated at 35 °C and 300 rev min^{-1} until reaching an optical density (OD) at 540 nm = 1.00. Cells were harvested by centrifugation, washed with ice-cold 50 mM MOPS, pH 7.4, centrifuged again, and resuspended in ice-cold 50 mM MOPS, pH 7.4 (0.25 g cells ml^{-1}). One milliliter cell suspension was sonicated (Vibra Cell Sonicator with microtip, Sonics and Materials, Danbury, CT) 6 × 10 s at a setting of 3.0. Cell debris were removed by centrifugation in a microcentrifuge at 4 °C for 1 min. Supernatant was transferred to a clean microcentrifuge tube and centrifuged at 4 °C for 10 min. This clarified supernatant was used for determinations of pyruvate dehydrogenase activity and protein concentration. Reactions consisted of 51 mM MOPS, pH 7.4; 0.20 mM magnesium chloride; 0.01 mM calcium chloride; 0.30 mM cocarboxylase; 0.12 mM coenzyme A; 2.0 mM B-nicotinamide adenine dinucleotide (B-NAD); 2.64 mM L-cysteine hydrochloride; 5.1 mM pyruvate; and clarified supernatant. A commercial PDHC preparation was used as the positive control (Cat. No. GL0124-010; GloboZymes, Carlsbad, CA). Pyruvate dehydrogenase activity was determined spectrophotometrically at 340 nm (Beckman DU 520 spectrophotometer, 1 cm path length, Beckman Coulter) at 22 °C as described (Brown and Perham, 1976). One unit of pyruvate dehydrogenase activity converted 1.0 mMol B-NAD to B-NADH min^{-1} (mg protein)$^{-1}$. Clarified supernatant for oxoglutarate dehydrogenase assays was obtained as for the pyruvate dehydrogenase assay. Oxoglutarate dehydrogenase reactions consisted of 50.8 mM MOPS, pH 7.4; 0.2 mM magnesium chloride; 0.01 mM calcium chloride; 0.3 mM cocarboxylase; 0.12 mM coenzyme A; 2.0 mM B-NAD; 2.6 mM L-cysteine; 5.0 mM α-ketoglutarate; and clarified supernatant. A commercial oxoglutarate dehydrogenase complex (OGDHC) preparation was used as positive control (Cat. No. K1502, Sigma Chemical Co., St. Louis, Mo). Oxoglutarate dehydrogenase activity was determined spectrophotometrically at 340 nm at 22 °C using conditions recommended by the supplier. One U of oxoglutarate dehydrogenase activity converted 1.0 mMol B-NAD to B-NADH min^{-1} (mg protein)$^{-1}$ in the presence of saturating levels of coenzyme A. Protein concentrations were determined by the method of Bradford (Bradford, 1976) with BSA as standard using procedures recommended by the manufacturer (BioRad Laboratories, Hercules, CA). The experiment was performed three times with each growth medium for pyruvate dehydrogenase and oxoglutarate dehydrogenase unit calculations. Results of all experiments were combined prior to analysis.

2.5. In vitro growth assays

To determine if *E. cloacae* M43 grew like an *ace* mutant on minimal medium + glucose (Langley and Guest, 1974), strains cultured overnight in M56 salts broth + 0.2% glycerol were harvested by centrifugation, washed twice

in 10 mM $MgSO_4$, and resuspended in 10 mM $MgSO_4$ to an OD of 1.00 at 540 nm. This suspension (500 μl) was added to 20 ml test treatment in 250 ml Erlenmeyer flasks. Test treatments were 50 mM glucose supplemented with nothing, or 50 mM glucose supplemented with 2 mM acetate, 2 mM lipoate, 2 mM lactate, 2 mM fumarate, 2 mM citrate, 2 mM L-glutamate, 2 mM L-aspartate, 2 mM succinate, or 2 mM L-lysine + 2 mM L-methionine. Flasks were incubated at 300 rev min^{-1} and 35 °C. Growth was monitored by periodically measuring the OD at 540 nm. Experiments were performed twice with two replicates per treatment and experiments were analyzed independently.

To determine if *E. cloacae* M43 grew in a similar fashion as *E. coli ace* mutants on reduced carbon compounds under anaerobic conditions, strains were incubated overnight, washed, and suspensions prepared as above. The suspension (10 μl) was applied to the perimeter of a M56 salts agar plate containing various reduced carbon treatments and quadrant streaked on the plate. Plates were incubated at 35 °C under anaerobic conditions in an anaerobe jar (GasPak 100 system, Becton Dickinson, Franklin Lakes, NJ) or under aerobic conditions as a control. Reduced carbon treatments are listed in Table 2. Populations in suspensions applied to plates were determined by dilution plating. The following rating system was used: 6+, discrete colonies after 1 d incubation; 5+, discrete colonies after 2 d incubation; 4+, discrete colonies after 3 d incubation; 3+, discrete colonies after 5 d incubation; 2+, discrete colonies after 9 d incubation; +, discrete colonies after 14 d incubation; NG, no growth, no discrete colonies after 14 d incubation. Two experiments were conducted with each reduced carbon source with two replicate plates per treatment. Experiments were analyzed independently.

To determine relative growth on various carbon sources detected in cucumber root exudate when supplied individually, *E. cloacae* strains were incubated overnight, washed, and resuspended as above, and 100 μl of this suspension added to 5 ml M56 basal salts + 0.2% test carbon source in sterile test tubes (1.8-cm diameter X 15 cm). Carbon sources supplied individually are listed in Table 3. Relative growth on 0.5%, 0.1%, and 0.05% synthetic root exudate, or synthetic root exudate plus 2 mM acetate, was determined as above using the mixture of reduced carbon compounds described by Roberts et al. (2007). Treatments were incubated at 35 °C and 250 rev min^{-1}. Experiments were performed at least three times with three replicates per treatment and experiments analyzed independently. Growth was monitored by determining the OD at 540 nm.

Table 2
Growth by *Enterobacter cloacae* strains under aerobic and anaerobic conditions on agar media containing different reduced carbon sources[a]

Reduced carbon source	Aerobic		Anaerobic	
	501R3	M43	501R3	M43
Glucose	6+	5+	3+	3+
Fructose	6+	4+	3+	3+
Acetate	5+	5+	NG	NG
Citrate	5+	NG	2+	+
Glutamine	5+	NG	NG	NG
Proline	5+	2+	NG	NG

[a]Strains containing standardized inoculum were streaked onto M56 basal salts agar containing 50 mM reduced carbon source and incubated at 35 °C under aerobic or anaerobic conditions. 6+, discrete colonies apparent after 1 d incubation; 5+, discrete colonies apparent after 2 d incubation; 4+, discrete colonies apparent after 3 d incubation; 3+, discrete colonies apparent after 5 d incubation; 2+, discrete colonies apparent after 9 d incubation; +, discrete colonies apparent after 14 d incubation; NG, no growth, no discrete colonies apparent after 14 d incubation.

2.6. Seed colonization experiments

E. cloacae strains were grown overnight in LB, washed, resuspended in sterile distilled water (SDW), and applied to single cucumber (*Cucumis sativum* cv. Marketmore 76), pea (*Pisum sativum* cv. Sugarsnap), sunflower (*Helianthus giganteus*), or wheat (*Triticum aestivum* cv. Jackson) seeds or sterile glass beads as 40 μl aqueous suspensions containing approximately 10^4 CFU as described previously (Roberts et al., 1992). Control treatments were seeds spotted with SDW only. Seeds were buried in 4 cm^3 soil-less mix (Premier Promix PGX, Quakertown, PA) in sterile

Table 3
In vitro growth by *Enterobacter cloacae* M43 on predominant carbohydrates, amino acids, and organic acids in cucumber root exudate

Compound[a]	*In vitro* growth[b]
Carbohydrate	
Cellobiose	NG
Galactose	NG
Glucose	NG
Mannose	NG
Rhamnose[c]	S
Sucrose	NG
Xylose	NG
Amino acid	
L-Aspartate	S
L-Glutamine	NG
L-Proline	S
L-Serine	NG
Organic acid	
Citrate	S
Malate	S
Succinate	S

[a]Carbohydrate, amino acid, and organic acid compounds tested were present at ⩾1 μg $plant^{-1}$ in cucumber root exudate (Liu et al., submitted). Only compounds that supported growth by strain 501R3 were tested.

[b]S, slow growth, OD at 540 nm of strain M43 was more than one standard deviation less than that of strain 501R3 at one or more time points. NG, no growth, OD at 540 nm was less than 0.10 at the end of the 6 h experiment for carbohydrates, with the exception of rhamnose, and less than 0.10 at the end of the 24 h experiment for amino acids and organic acids.

[c]Growth by rhamnose was evaluated over 24 h. Neither strain 501R3 nor M43 had an OD at 540 nm greater than 0.15 after 6 h.

snap-cap tubes and incubated at 22 °C. Soil-less mix had been previously equilibrated with 1 ml SDW. Seeds were not surface sterilized. CFU were determined at various times by sonicating (Model 8210, Branson Ultrasonics Corp., Danbury, CT) and subsequently dilution-plating the entire contents of the tubes onto LB agar containing the appropriate antibiotics. The mean $\log_{10}$ CFU seedling^{-1} for each strain was determined, ANOVA carried out, and differences among means estimated using Duncan's Multiple Range Test (SAS, Cary, NC). Treatments were replicated six times and the experiment was performed twice. Experiments were analyzed independently.

2.7. Root colonization experiments

Nonsurface-sterilized cucumber, pea, sunflower, and wheat seeds, treated with approximately 10^8 CFU seed^{-1} of *E. cloacae* strains in a gelatin formulation as described (Roberts et al., 2005), were sown in a natural Hatborough loamy sand soil (pH 4.5, 0.6% humic matter) or in soil-less mix in 6.5-cm diameter X 25-cm deep pots and incubated in a growth chamber at 22 °C with a 12-h photoperiod. Control treatments were seeds treated with the gelatin formulation but no bacteria. Plants were removed at sampling time and the entire root system sampled by cutting the root at the soil line. The entire root system and adhering planting medium were placed in SDW, sonicated and CFU determined as described (Roberts et al., 1997). Root system fresh weight was determined for each sample. Log_{10} CFU (g fresh weight root tissue)$^{-1}$ was determined for each treatment and compared using the Student's *t* test (SAS). The experiment was performed two times with six replicates per treatment for each crop species × planting medium combination. Experiments were analyzed independently.

3. Results

3.1. Molecular and biochemical characterization of E. cloacae M43

E. cloacae M43 was isolated after screening a library of mini-Tn*5* Km transposon mutants derived from wild-type strain 501R3 for mutants that failed to grow on minimal media containing reduced carbon compounds detected in seed and root exudate. Southern hybridization analysis demonstrated the presence of a single mini-Tn*5* Km insertion in the genome of strain M43 (data not shown) and a portion of the M43 genome containing this transposon was subcloned in pM43. Sequencing outward from mini-Tn5 Km in pM43, with primers specific for the ends of mini-Tn*5* Km, indicated that this transposon was inserted in a region of the strain M43 genome with a high degree of similarity to *aceF* (Table 1, Fig. 1), which encodes the dihydrolipoamide acetyltransferase subunit (E2) of the PDHC (Keseler et al., 2005).

Plasmid pM43 contains partial sequences of two genes, the 3′ end of *aceE* (pyruvate dehydrogenase, E1) and the 5′ end of *aceF*. The completed sequence of *aceF*, the 5′ end of *lpd*, and the intergenic sequence between these genes were obtained by sequencing PCR product generated from reactions using primers anchored in *aceE*, *aceF*, and *lpd* (Fig. 1). Available *aceF* sequences from strain 501R3, strain M43, and pM43 were identical and the mini-Tn*5* Km insertion site was identical in both pM43 and genomic DNA isolated from strain M43. The combined 3474-bp sequence contained (1) 1190-bp of the 3′ terminus of the *aceE* gene, which encodes the last 395 amino acids of subunit E1; (2) the complete sequence of *aceF* (1893-bp), sufficient to encode the 630 amino acids of subunit E2; and (3) the first 18 codons of the 5′ terminus of *lpd*, which encodes the lipoamide dehydrogenase subunit E3. At the protein level, *aceF*, the only complete ORF in the sequence, showed between 88.9% to 92.2% identity and between 95.1% to 95.8% similarity when compared to all the *E. coli* strains included in this analysis, *Shigella flexneri* 2a 301, *Sh. sonnei* Ss046 and *Erwinia caratovora* subsp. *atroseptica*. It showed 68.2% identity and 76.9% similarity when compared to *Yersinia pseudotuberculosis* IP32953. A ribosome binding site, GAGGTA, was detected at positions 1191–1196 in the 14-bp intergenic sequence between *aceE* and *aceF*. This intergenic region is identical in other Enterobacteriaceae representatives, including *E. coli* strains K12 MG1655, W3110, APEC01, UTI89, and O157:H7 EDL933, *Salmonella typhimurium* LT2, *S. enterica* CT18, *Sh. boydii* Sb227, *Sh. flexneri* 2a 301, and *Sh. sonnei* Ss046. The RBS is conserved in *E. caratovora* subsp. *atroseptica* SCRI1043 and *Y. pseudotuberculosis* IP32953, although the rest of the 14-bp sequence shows some variation. The *aceF* transcription terminus was located between positions 3130 and 3141, in the intergenic region between *aceF* and *lpd* in *E. cloacae*. This region of *E. cloacae* is identical in all *E. coli* strains studied (K12 MG1655, W3110, APEC01, UTI89, O157:H7 EDL933), *Sh. flexneri* 2a 301, *Sh. sonnei* Ss046 and *E. caratovora* subsp. *atroseptica*. These sequences differ considerably in *S. typhimurium* LT2, *S. enterica* CT18 and *Y. pseudotuberculosis* IP32953. A promoter sequence (P*lpd*) was identified downstream of the *E. cloacae aceF* terminus, at positions 3190 to 3219. This *lpd* promoter shows high levels of conservation (greater than 96% identity in 30 nucleotides) in all analyzed strains of *E. coli* (K12 MG1655, W3110, APEC01, UTI89, O157:H7 EDL933), as well as *S. typhimurium* LT2, *S. enterica* CT18, *Sh. boydii* Sb227, *Sh. flexneri* 2a 301, *Sh. sonnei* Ss046, and *E. caratovora* subsp. *atroseptica* SCRI1043. It differs considerably in *Y. pseudotuberculosis* IP32953. The transcription start site of *lpd* in *E. cloacae* was located at position 3239, which is 191-bp upstream from the *lpd* initiation codon. The 3474-bp sequence obtained for *E. cloacae* most closely matched those of *E. coli* strains K12 MG1655 and W3110, showing 90 and 92% DNA identity, respectively. A gene context analysis showed a remarkably conserved physical organization of

the *aceEF* and *lpd* genes in several, but not all, representatives of the Beta and Gammaproteobacteria subdivisions. This organization is not present in the Alpha, Delta and Epsilon subdivisions of Proteobacteria nor in any other bacterial group, including Archaeobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Chlamidiae, and Cyanobacteria.

The mini-Tn5 Km insertion in strain M43 significantly reduced pyruvate dehydrogenase activity, as expected for an *aceF* mutant. Pyruvate dehydrogenase activity in cell lysates of strain 501R3 grown on 50 mM glycerol + 2 mM acetate was 0.011 ± 0.0036 U while that of strain M43 grown on this medium was below detectable limits. Pyruvate dehydrogenase activity was reduced in cell lysates from strain 501R3 grown on 50 mM glucose + 2 mM acetate probably due to catabolite repression (Guest and Russell, 1992). Pyruvate dehydrogenase activity in these cell lysates was $5.74 \pm 1.70 \times 10^{-3}$ U. There was no detectable pyruvate dehydrogenase activity in cell lysates from strain M43 grown on 50 mM glucose + 2 mM acetate. *lpd* encodes the lipoamide dehydrogenase subunit of PDHC and OGDHC in *E. coli* (Keseler et al., 2005) and is immediately downstream from *aceF* in *E. cloacae* (Fig. 1) and in *E. coli*. *lpd* can be transcribed from the strong *aceE* promoter and from the weak *lpd* promoter in *E. coli* (Spencer and Guest, 1985; Keseler et al., 2005). Assays of oxoglutarate dehydrogenase activity in cell lysates from strains 501R3 and M43 indicated that the mini-Tn*5* Km insertion in *aceF* did not affect activity of this enzyme complex when strain M43 was grown in 50 mM glycerol + 2 mM acetate. Oxoglutarate dehydrogenase activity was $3.74 \pm 1.89 \times 10^{-3}$ and $3.42 \pm 0.98 \times 10^{-3}$ U for strains 501R3 and M43, respectively, grown in 50 mM glycerol + 2 mM acetate. Oxoglutarate dehydrogenase activity was below detectable limits for both strains when grown on 50 mM glucose + 2 mM acetate. It is possible that *lpd* was transcribed from P*lpd* in strain M43 grown on 50 mM glycerol and 2 mM acetate, allowing wild-type oxoglutarate dehydrogenase activity.

Acetate (2 mM) was required as a supplement for detectable aerobic growth (in 7 h experiments) by strain M43 on minimal media containing 50 mM glucose as the primary source of carbon and energy while 50 mM glucose without a supplement supported substantial growth by wild-type strain 501R3 over 7 h (Fig. 2). Glucose (50 mM) supplemented with 2 mM lipoate, 2 mM lactate, 2 mM fumarate, 2 mM citrate, 2 mM L-glutamate, 2 mM L-aspartate, 2 mM succinate, or 2 mM L-lysine + 2 mM L-methionine did not support growth of strain M43 in a 7 h experiment. These results are similar to those of Langley and Guest (1974) with an *ace* mutant of *E. coli*. Growth by strain M43 on 50 mM glucose + 2 mM acetate was not simply due to the presence of additional substrate in the growth medium as strain M43 failed to grow on 52 mM glucose (data not shown) or on 50 mM glucose supplemented with 2 mM compounds listed above (with the exception of acetate). Some of these compounds can serve as the sole source of carbon and energy for *E. cloacae*

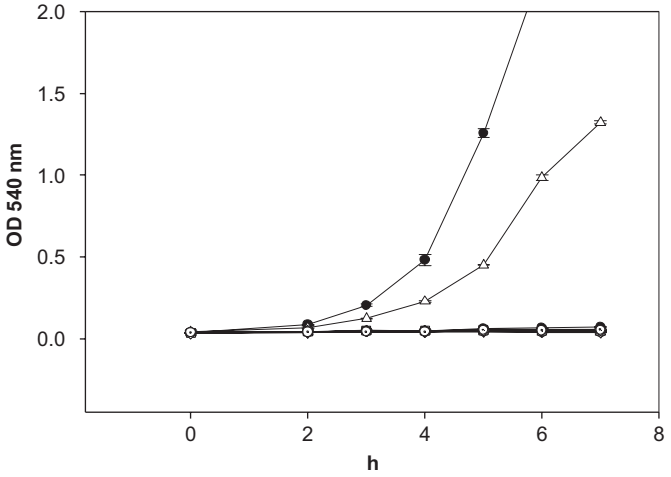

Fig. 2. Growth by *Enterobacter cloacae* strains 501R3 and M43 on M56 minimal salts medium plus 50 mM glucose and 2 mM supplement. Closed circle, 501R3 grown on 50 mM glucose only; open circle M43 grown on glucose only; Closed dotted circle, 501R3 grown on 50 mM acetate only; open dotted circle, M43 grown on 50 mM acetate only. Strain M43 was also grown on 50 mM glucose supplemented with: 2 mM acetate (open upward arrow), lipoate (open downward arrow), lactate (open diamond), fumarate (open hexagon), citrate (open box), glutamate (open dotted box), aspartate (open dotted upward arrow), succinate (open dotted downward arrow), lysine and methionine (open dotted diamond). Mean optical density of two replicate samples with standard deviation over time is shown.

(Roberts et al., 1992). It should be noted that growth by strain M43 on these substrates was apparent after overnight incubation (data not shown). Similar results were obtained in repeated experiments (data not shown). Growth by strain M43 on glucose and fructose was similar to that by strain 501R3 and slower than that by strain 501R3 on citrate under anaerobic conditions (Table 2), as expected for an *ace* mutant (Langley and Guest, 1974). Similar results were obtained in a second experiment (data not shown). Wild-type growth on glucose and fructose under anaerobic conditions was probably due to pyruvate formate lyase activity as with other enteric bacteria (Cassey et al., 1998).

3.2. *In vitro* growth on reduced carbon compounds detected in cucumber root exudate

E. cloacae strain M43 was significantly reduced in growth on all of the predominant carbohydrates, amino acids, and organic acids detected in cucumber root exudate when these compounds were supplied individually (Table 3). Similar results were obtained in two additional experiments (data not shown). Relative growth of *E. cloacae* strains M43 and 501R3 was also determined on synthetic cucumber root exudate supplied at various final concentrations. This synthetic root exudate contained 14 carbohydrates, four TCA cycle intermediates, and 17 amino acids in the relative proportions they were detected at in cucumber root exudate (Roberts et al., 2007). Strain M43 was dramatically reduced in growth relative to strain

501R3 on this synthetic cucumber root exudate when total reduced carbon was supplied at 0.5% (Fig. 3), 0.1%, and 0.05% (data not shown) in M56 basal salts medium. Addition of 2 mM acetate slightly increased growth by strain M43 at all concentrations of the growth medium relative to growth by this strain without the acetate supplement. However, the addition of acetate did not increase growth by strain M43 to levels associated with strain 501R3. Similar results were obtained in repeated experiments (data not shown).

3.3. Seed and root colonization

E. cloacae M43, with a mutation in *aceF*, was substantially reduced in short-term colonization ability on cucumber, pea, sunflower, and wheat seeds (Table 4). Populations of *E. cloacae* 501R3, the parental strain, increased substantially and were significantly greater ($P \leqslant 0.05$) than those of strain M43 at 48 h and 72 h on all seed types. Populations of *E. cloacae* M43 increased significantly on only pea seed over 72 h. Similar results were obtained in a second experiment (data not shown).

E. cloacae M43 was substantially impaired in long-term colonization of roots of cucumber relative to strain 501R3 (Table 5). Populations of strain M43 were at least 580-fold lower than those of strain 501R3 at one or more time points in experiments conducted with two different planting media. *E. cloacae* M43 was also substantially impaired in long-term colonization of roots of pea, sunflower, and wheat relative to strain 501R3 (Table 6). Populations of strain M43 ranged between 630-fold lower and 93,000-fold lower than those of strain 501R3 in the rhizospheres of pea, sunflower, and wheat after 42 d in two experiments conducted in soil-less mix. Similar populations of strains 501R3 and M43 (approximately 10^8 CFU seed^{-1})

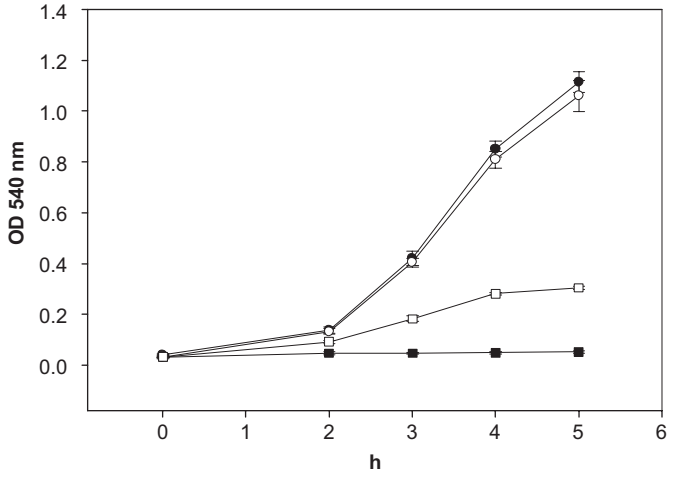

Fig. 3. Growth by *Enterobacter cloacae* strains 501R3 and M43 on M56 minimal salts medium plus 0.5% synthetic cucumber root exudate with and without 2 mM acetate. Circles, strain 501R3; squares, strain M43. Closed symbols, no acetate added; open symbols, 2 mM acetate added. Mean optical density of three replicate samples with standard deviation over time from a single experiment is shown.

Table 4
Growth of *Enterobacter cloacae* 501R3 and M43 on various seeds or glass beads in soil-less mix[a]

Seed	Time (h)	Log_{10} CFU seed^{-1}	
		501R3	M43
Cucumber	0	3.99 HBL	3.83 IBL
	24	4.01 HBL	3.69 KLM
	48	5.31 EF	3.56 LM
	72	5.26 EF	3.09 M
Pea	0	4.04 HBL	3.91 HBL
	24	4.51 GH	3.99 HBL
	48	6.48 BC	4.94 FG
	72	7.21 A	5.96 CD
Sunflower	0	3.99 HBL	3.76 JKL
	24	4.05 HBL	3.47 LM
	48	6.56 B	3.88 HBL
	72	6.77 AB	4.50 HIJ
Wheat	0	4.04 HBL	3.77 JKL
	24	4.49 GHI	3.87 HBL
	48	5.66 DE	4.27 HBK
	72	5.84 DE	3.81 JKL
Glass bead	0	4.02 HBL	3.85 HBL
	24	3.94 HBL	3.66 KLM
	48	4.40 GBJ	3.45 LM
	72	4.32 GBK	3.51 LM

[a]Bacteria were applied to seed as a 40 µl suspension in sterile distilled water. Populations of bacteria were determined at 0, 24, 48, and 72 h by dilution plating. Values are the mean of six replicate samples from a single experiment. Comparisons were made between populations on all seed types at all times. Means followed by the same letter are not significantly different ($P \leqslant 0.05$).

Table 5
Long-term colonization of cucumber roots by *E. cloacae* 501R3 and M43[a]

Planting medium	Experiment	Time	Log_{10} CFU (g fresh weight root)$^{-1}$		*P*
			501R3	M43	
Natural soil	1	21 d	5.48	3.36	0.0008*
		42 d	5.25	2.48	<0.0001*
	2	27 d	5.62	BDT[b]	<0.0001*
		42 d	5.64	BDT	<0.0001*
Soil-less mix	1	25 d	5.03	4.43	0.43
		40 d	5.24	1.65	0.0002*
	2	25 d	4.84	BDT	<0.0001*
		40 d	5.84	BDT	<0.0001*

[a]Similar populations (approximately 10^8 CFU seed^{-1}) of strains 501R3 and M43 were applied to cucumber seeds in a gelatin formulation. Populations were determined by dilution plating the entire root system and tightly adhering planting medium. Values represent the mean log_{10} CFU (g fresh weight root)$^{-1}$ from six replicate samples. Asterisks indicate statistically significant differences ($P \leqslant 0.05$) at that time point in that particular experiment.

[b]BDT, below detectable threshold. No colonies were evident on agar plates after dilution plating.

Table 6
Long-term colonization of pea, sunflower, and wheat roots by *E. cloacae* 501R3 and M43 in experiments conducted in soil-less mix[a]

Crop	Experiment	Log_{10} CFU (g fresh weight root)$^{-1}$		*P*
		501R3	M43	
Pea	1	5.22	0.25	<0.0001*
	2	4.92	BDT[b]	<0.0001*
Sunflower	1	4.73	1.93	0.028*
	2	4.82	0.30	0.0002*
Wheat	1	5.18	1.87	0.0067*
	2	4.77	BDT	<0.0001*

[a]Similar populations (approximately 10^8 CFU seed^{-1}) of strains 501R3 and M43 were applied to all crop seeds in a gelatin formulation. Populations of these strains were determined at 42 d by dilution plating the entire root system and tightly adhering soil-less mix. Values represent the mean log_{10} CFU (g fresh weight root)$^{-1}$ from six replicate samples. Asterisks indicate statistically significant differences ($P \leqslant 0.05$) at 42 d on that crop in that particular experiment.

[b]BDT, below detectable threshold. No colonies were evident on agar plates after dilution plating.

were added to seeds in a gelatin formulation in all long-term colonization experiments.

4. Discussion

E. cloacae M43, a mini-Tn*5* Km transposon mutant derivative of *E. cloacae* 501R3, was dramatically impaired in utilization of reduced carbon compounds in seed and root exudate and in colonization of seeds and roots of diverse crop plants (Fig. 3, Tables 3–6). Genetic and biochemical data presented here demonstrate that the mutation in *E. cloacae* M43 is in *aceF*, a gene encoding a subunit of PDHC. (1) *E. cloacae* M43 contains a sole mini-Tn*5* Km insertion within a region of the *E. cloacae* genome with very high DNA sequence identity to *aceF* from *E. coli* (data not shown), (2) *E. cloacae* M43 was substantially reduced in pyruvate dehydrogenase activity relative to strain 501R3, and (3) *E. cloacae* M43 requires acetate as a nutritional supplement for vigorous aerobic growth on glucose, as expected for an *ace* mutant (Langley and Guest, 1974). *lpd*, which encodes lipoamide dehydrogenase, is located downstream of *aceF* in *E. coli* (Keseler et al., 2005) and *E. cloacae* (Fig. 1). Expression of *lpd* is driven by P*aceE* and the weaker P*lpd* promoters in *E. coli* (Keseler et al., 2005) and possibly in *E. cloacae* and other Gamma and Betaproteobacteria. Lipoamide dehydrogenase is a subunit component of both the PDHC and OGDHC. However, the similarity in oxoglutarate dehydrogenase activity between strains M43 and 501R3 suggests that the nutritional loss profile of strain M43 and the reduction in colonization of plant seeds and roots by strain M43 was solely due to the loss of pyruvate dehydrogenase activity.

The dramatic decrease in colonization of seeds and roots by strain M43 was due to the lost ability of this strain to grow on carbohydrates, amino acids, and organic acids in exudate and not due to auxotrophy. Strain M43 was a prototroph requiring acetate as a supplement only for vigorous aerobic growth on glucose. Aerobic growth on minimal salts broth plus glucose by strain M43 in the absence of acetate was detected after overnight incubation (data not shown). Colonies of strain M43 were evident after 2 or 3 d incubation under aerobic conditions on minimal salts agar plus glucose, fructose, or acetate and anaerobic growth on glucose by strain M43 was similar to that of strain 501R3 (Table 2). The dramatic decrease in colonization of seeds and roots by strain M43 was also not due to PDHC functioning as a DNA-binding regulatory molecule as PDHC has no such known role.

The diminished nutritional capabilities of strain M43 were in essence a combination of the diminished nutritional capabilities of *E. cloacae* strains A-11 and M2. Strain M43 was severely impaired in growth on almost all carbohydrates, and on almost all amino acids and organic acids detected in cucumber root exudate (Table 3, Fig. 3). *E. cloacae* A-11 was severely impaired in growth on almost all carbohydrates detected in seed and root exudate as a result of a single transposon insertion in *pfkA* but essentially unaffected in growth on organic acids and amino acids (Roberts et al., 1999; Liu et al., submitted). *E. cloacae* M2 was severely impaired in growth on almost all amino acids and organic acids detected due to a single transposon insertion in *sdhA* but relatively unaffected in growth on the carbohydrates (Liu et al., submitted). Although not conducted concurrently, seed colonization and cucumber root colonization experiments presented here with *E. cloacae* M43 were performed under similar conditions as seed and root colonization experiments conducted with *E. cloacae* strains A-11 and M2 (Roberts et al., 1996, 1999; Liu et al., submitted). This allows comparison of the relative colonization behavior of strain M43 with that of strains A-11 and M2. In seed colonization experiments, strain M43 was significantly reduced in colonization of pea seed and deficient in colonization on all other seed types tested relative to strain 501R3 (Table 4). In contrast, strains A-11 and M2 were reduced in colonization on some, but not all seeds tested relative to strain 501R3 (Roberts et al., 1996, 1999). The mutation in strain M43 also resulted in a dramatically larger impact on cucumber root colonization than either the mutation in strain A-11 or in strain M2. Strain M43 was essentially deficient in colonization of cucumber rhizosphere with populations of this strain below detectable limits in several experiments (Table 5) while strains A-11 and M2 were only slightly reduced in cucumber root colonization relative to strain 501R3 in experiments where bacteria were applied to cucumber seed above carrying capacity (Liu et al., submitted). Taken together, these experiments demonstrate considerable nutritional flexibility by *E. cloacae* in the spermosphere and rhizosphere. Only when *E. cloacae* is deficient in growth on almost all nutrients, as is the case for strain M43, is colonization dramatically impacted on all crops tested.

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